

Campylobacter volucris sp. nov., isolated from black-headed gulls (*Larus ridibundus*)

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During a study of the prevalence of *Campylobacter jejuni* in black-headed gulls (*Larus ridibundus*) in Sweden, three isolates, strains LMG 24379, LMG 24380^T and LMG 24381, were initially identified as *Campylobacter lari*. Further characterization by both AFLP and whole-cell protein SDS-PAGE analyses revealed that they formed a distinct group in the genus *Campylobacter*. This unique position was confirmed by phenotypic characterization, 16S rRNA and *hsp60* gene sequence analysis and DNA–DNA hybridizations. The combined data confirm that these isolates represent a novel species within the genus *Campylobacter*, for which the name *Campylobacter volucris* sp. nov. is proposed. The type strain is LMG 24380^T (=CCUG 57498^T).

The genus *Campylobacter*, belonging to the class *Epsilonproteobacteria*, was proposed by Sebald & Véron (1963) to accommodate two *Vibrio*-like species, currently known as *Campylobacter fetus* and *Campylobacter sputorum*. The genus has expanded steadily since then and, at the time of writing, comprises 21 species and 8 subspecies (Debruyne *et al.*, 2008, 2009; Rossi *et al.*, 2009; Zannoni *et al.*, 2009). Over the years, a strong association has been demonstrated between certain *Campylobacter* species and birds, both domesticated and wild. Some of these *Campylobacter* species, like *Campylobacter canadensis*, have so far been isolated solely from birds (Inglis *et al.*, 2007), while others, like *Campylobacter jejuni*, can be found in avian and human and other mammalian sources (Broman

et al., 2002, 2004; Petersen *et al.*, 2001; Waldenström *et al.*, 2002, 2007). The isolation of campylobacteria from wild birds, including species that are zoonotic enteropathogens, indicates that wild birds potentially serve as a reservoir for human infection. A limited number of studies have focused on this epidemiological aspect, in particular for *C. jejuni*. One report describes the isolation from wild peregrine falcons in Sweden in 2000 of two *C. jejuni* isolates that shared identical PFGE patterns with an isolate obtained from a young man in southern Sweden in 1999 (Palmgren *et al.*, 2004). Genotyping by multilocus sequence typing (Colles *et al.*, 2009; French *et al.*, 2009) and PFGE (Broman *et al.*, 2002, 2004) of a large selection of *C. jejuni* isolates, originating from wild birds, domestic animals and humans, showed that strains isolated from wild birds are most often different from clinical strains but that similarities occur in some cases, notably in birds strongly associated with human activities. Other studies also seem to indicate that wild animals and birds carry their own, host-species-adapted *Campylobacter* populations, distinct from those found in domesticated animals and humans (Petersen *et al.*, 2001; Waldenström *et al.*, 2007).

Black-headed gulls (*Larus ridibundus*) belong to the ecological guild of the opportunistic feeders, a group that was reported to be among the ecological guilds of birds most commonly infected by campylobacteria (Waldenström *et al.*,

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *hsp60* gene sequences of strains LMG 24379, LMG 24380^T and LMG 24381 are FM883693–FM883695 (16S rRNA gene) and FM883696–FM883698 (*hsp60*), respectively.

Dendrograms based on AFLP fingerprints and whole-cell protein SDS-PAGE profiles and a table of differential properties for the novel species and all members of *Campylobacter* are available as supplementary material with the online version of this paper.

2002). They are omnivorous and feed in natural habitats, as well as in farmland and urban surroundings. During a study on the prevalence of *C. jejuni* in this bird species in southern Sweden (Broman *et al.*, 2002), three strains, originating from three individual birds, were isolated that gave a negative reaction in the *C. jejuni*/*C. coli* multiplex PCR (Vandamme *et al.*, 1997) that was used for species identification. Subsequently, these isolates were subjected to 23S rRNA PCR-RFLP (Fermer & Engvall, 1999) and were identified as *Campylobacter lari*. During a large-scale investigation of the diversity of *C. lari*-like bacteria, these three isolates formed a distinct group, by both whole-cell protein SDS-PAGE and AFLP analysis. In the present study, their unique position was further examined by determination of phenotypic characteristics, 16S rRNA and *hsp60* gene sequence analysis and DNA–DNA hybridizations.

Details of the sampling procedure were described previously (Broman *et al.*, 2002). Sampling was performed in Malmö, southern Sweden, in March 1999. Birds were sampled by insertion of a sterile swab in the cloaca, and each swab was placed in Amies charcoal transport medium (Transwab; BioDisc) and cultured within 24–72 h of sampling. The samples were plated onto *Campylobacter* selective medium, consisting of (l^{-1}) 42.5 g Columbia blood agar base (Acumedia Manufacturers), 5 % defibrinated horse blood, 10 mg vancomycin, 2500 IU polymyxin B and 5 mg trimethoprim, and incubated at 42 °C under microaerobic (5 % O₂, 10 % CO₂, 85 % N₂) conditions. The plates were checked for *Campylobacter*-like growth after 24 and 48 h of incubation. From each plate with growth of suspected campylobacters, one colony was subcultured on blood-agar plates and investigated further. Isolates were stored at –80 °C in trypticase soy broth (BD) supplemented with 15 % glycerol.

Strains were subsequently cultured on Mueller–Hinton agar (Oxoid), supplemented with 5 % sterile horse blood, and incubated at 37 °C for 48 h under microaerobic conditions. DNA was extracted as described by Pitcher *et al.* (1989).

AFLP analysis was performed as described previously (Debruyne *et al.*, 2009). In brief, 1 µg genomic DNA was digested with the restriction enzyme combination *Hind*III/*Hha*I, after which site-specific adapters were ligated to the restriction fragments. Primers complementary to the adaptor and restriction site sequence were used in subsequent preselective and selective PCR amplification reactions. The amplified and fluorescently labelled fragments were loaded on a denaturing polyacrylamide gel on an ABI Prism 377 automated sequencer. GENESCAN version 3.1 (Applied Biosystems) was used for data collection, and the generated profiles were imported, using the CrvConv filter, in BioNumerics version 4.61 (Applied Maths) for normalization and further analysis. After normalization, the AFLP profiles were imported into an in-house AFLP reference database that contains profiles from type and reference strains of all established *Campylobacter* species.

The similarity between profiles was determined by Pearson's correlation coefficient, and cluster analysis was performed by the unweighted pair group method with arithmetic means (UPGMA). AFLP profiles of the three isolates representing the distinct taxon were divergent from those of other *C. lari*-like strains and all other *Campylobacter* species (Supplementary Fig. S1, available in IJSEM Online).

This divergence was supported by whole-cell protein SDS-PAGE analysis. Protein extraction and SDS-PAGE were performed as described by Pot *et al.* (1994). Similarity of the obtained normalized SDS-PAGE patterns was determined by Pearson's correlation coefficient, and clustering was performed by UPGMA, using BioNumerics version 4.61. For numerical analysis, a variable region of dense bands (36.1–43.2 kDa) (Vandamme *et al.*, 1990) was excluded to increase species discrimination. The results of the numerical analysis, in combination with visual inspection of the SDS-PAGE patterns, demonstrated that the SDS-PAGE patterns of the three novel strains were distinct from those of *C. lari* and of all other known *Campylobacter* species (Supplementary Fig. S2).

To determine the phylogenetic position of this taxon, 16S rRNA and *hsp60* gene sequences of isolates LMG 24379, LMG 24380^T and LMG 24381 were generated. *hsp60* gene sequences were determined as they offer improved resolution compared with 16S rRNA gene sequences (Kärenlampi *et al.*, 2004). 16S rRNA gene sequences were determined as described previously (Vandamme *et al.*, 2006). Sequences were assembled using BioNumerics version 5.1, and all three strains had identical 16S rRNA gene sequences. Comparison by the megablast algorithm to the GenBank sequence database revealed that the nearest phylogenetic neighbours were *C. lari* subsp. *concheus*, *C. lari* subsp. *lari*, *C. jejuni*, *C. coli*, *Campylobacter insulaenigræ*, *Campylobacter peloridis* and *Campylobacter subantarcticus*, all with similarity levels exceeding 97 %. For tree reconstruction, sequences were aligned using the CLUSTAL_X software package (Thompson *et al.*, 1997), and clustering was performed by the neighbour-joining method (Saitou & Nei, 1987) using BioNumerics version 5.1. Unknown bases were discarded for the analysis. Bootstrap values were determined using 500 replicates (Fig. 1). Partial *hsp60* gene sequencing was performed as described previously (Debruyne *et al.*, 2009), and further analysis was performed as for the 16S rRNA gene sequences (Fig. 2). The three isolates representing the novel taxon shared identical *hsp60* gene sequences (100 % sequence similarity), while inter-specific sequence similarities were no higher than 93.7 %.

Over 60 phenotypic characteristics were determined using an array of well-standardized methods described previously (On *et al.*, 1996). Results were compared with those for other *Campylobacter* species, and an overview of the differentiating characteristics between the novel species and its nearest phylogenetic neighbours (as determined by 16S rRNA gene sequence analysis) is provided in Table 1.

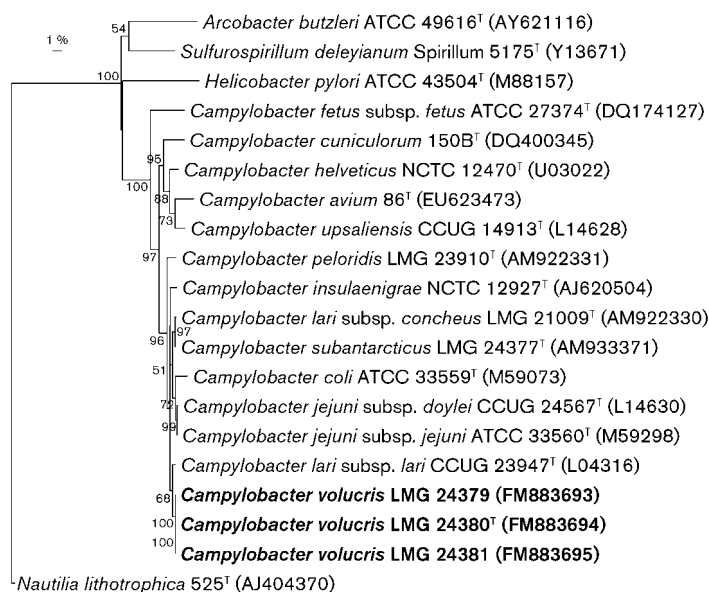


Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences constructed by the neighbour-joining method. Bootstrap values (%) are indicated at nodes. Bootstrap values lower than 50 % are not shown. Bar, 1 % sequence divergence.

Characteristics that allow the differentiation of the novel species from all other *Campylobacter* species are presented in Supplementary Table S1. The novel species only differs by a single phenotypic characteristic from both *Campylobacter lari* subsp. *concheus* (resistance to cepoparezone) and *C. canadensis* (resistance to cephalothin). However, they can be readily distinguished by whole-cell protein SDS-PAGE analysis or by analysis of the AFLP patterns.

For the determination of G+C content, DNA was enzymically degraded into nucleosides as described by Mesbah & Whitman (1989). The nucleoside mixture was separated by HPLC using a Waters SymmetryShield C8 column maintained at 37 °C. The solvent was 0.02 M (NH₄)₂HPO₄ (pH 4.0) with 1.5 % acetonitrile. Non-methylated λ phage

DNA (Sigma) was used as the calibration reference. The DNA G+C content of strain LMG 24380^T was 29 mol%, which falls just within the range reported for the genus *Campylobacter* (29–47 mol%; Debruyne *et al.*, 2008). An overview of G+C contents for *Campylobacter* species is given in Supplementary Table S1.

DNA–DNA hybridizations were performed between strain LMG 24380^T and the type strains of its closest relatives. DNA was extracted from 0.25–0.5 g (wet weight) cells as described by Pitcher *et al.* (1989). DNA–DNA hybridizations were performed with photobiotin-labelled probes in microplate wells (Ezaki *et al.*, 1989), using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridization temperature was 30 °C.

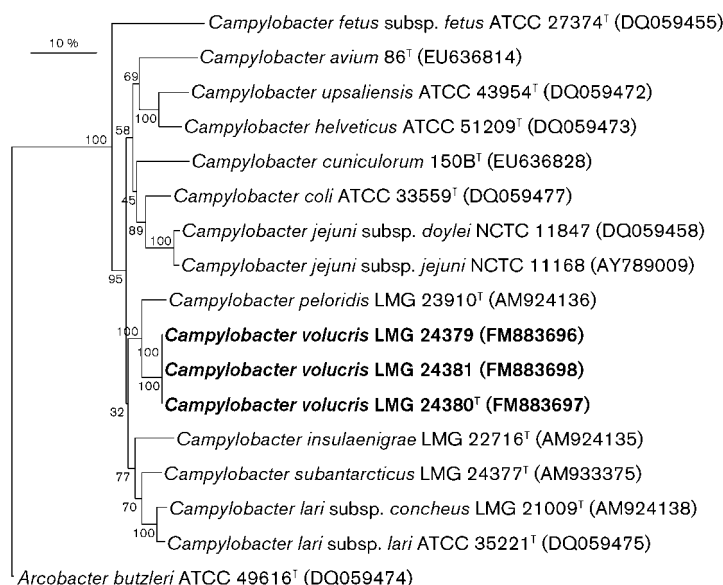


Fig. 2. Neighbour-joining tree based on partial *hsp60* gene sequences. All sequences were 555 bp in length, with the exception of the sequences for *C. cuniculorum* 150B^T and *C. avium* 86^T, which were respectively 489 and 499 bp in length. Bootstrap values (%) are indicated at nodes. Bar, 10 % sequence divergence.

Table 1. Differentiating characteristics between the novel strains and recognized members of the genus *Campylobacter*

Taxa: 1, *Campylobacter volucris* sp. nov. ($n=3$); 2, *C. coli*; 3, *C. insulaenigrae*; 4, *C. jejuni* (merged data for *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*); 5, *C. lari* subsp. *concheus*; 6, *C. lari* subsp. *lari*; 7, *C. peloridis*; 8, *C. subantarcticus*. Data for reference taxa were taken from the original descriptions (Foster *et al.*, 2004; Debruyne *et al.*, 2009, 2010) and from Logan *et al.* (2000), On *et al.* (1996, 1998) and Vandamme *et al.* (1991) and unpublished data of one of the authors (S.L.W.O.). +, Positive; w, weakly positive; (+), 68–95 % strains positive; v, 34–67 % strains positive; (–), 5–33 % strains positive; –, negative; NA, no data available. All taxa are positive for oxidase production.

Characteristic	1	2	3	4	5	6	7	8
Catalase production	+	+	+	(+)	+	+	+	+
Hippurate hydrolysis	–	–	–	(+)	–	–	–	–
Indoxyl acetate	–	+	–	+	NA	–	NA	–
Nitrate reduction	+	+	–	v	NA	+	NA	+
Growth at/on:								
42 °C	+	+	–	v	+	+	+	+
MacConkey agar	w	v	–	(–)	v	(+)	–	v
Nutrient agar	–	+	v	+	+	(–)	+	–
Growth in presence of:								
NaCl (2 %)	–	–	–	–	+	v	(+)	+
Glycine (1 %)	–	(+)	–	v	(+)	+	+	+
Safranin (0.02 %)	–	+	(–)	v	–	+	–	–
Sodium deoxycholate (0.1 %)	w	+	–	v	v	+	v	–
Nalidixic acid (32 mg ml ^{–1})	+	–	+	–	–	(+)	(+)	+
Cephalothin (32 mg ml ^{–1})	+	+	+	v	+	(+)	(–)	–
Metronidazole (4 mg ml ^{–1})	+	(+)	+	v	+	+	(+)	v
Carbenicillin (32 mg ml ^{–1})	+	(+)	+	v	+	+	–	–
Cefoperazone (64 mg ml ^{–1})	–	+	+	v	NA	+	NA	–
DNA G + C content (mol%)	29	30–33	NA	30–33	30	30–32	29	30

Reciprocal experiments were performed for every pair of strains and standard deviations ranged from 0.5–16.8 %. DNA–DNA hybridization values between strain LMG 24380^T and *C. lari* subsp. *lari* LMG 8846^T, *C. lari* subsp. *concheus* LMG 21009^T, *C. peloridis* LMG 23910^T, *C. subantarcticus* LMG 24377^T, *C. jejuni* subsp. *jejuni* LMG 8841^T, *C. coli* LMG 6440^T and *C. insulaenigrae* LMG 22716^T were respectively 40, 41, 37, 30, 24, 15 and 43 %. All these values are well below the threshold of 70 % recommended for species delineation (Stackebrandt & Goebel, 1994).

The present study demonstrates that the three isolates from black-headed gulls obtained during a sampling campaign in Malmö, southern Sweden, in March 1999 represent a novel species within the genus *Campylobacter* which can be distinguished from other *Campylobacter* species by AFLP fingerprinting (Supplementary Fig. S1), *hsp60* gene sequence analysis (Fig. 2), whole-cell protein electrophoresis (Supplementary Fig. S2) and biochemical characteristics. We propose to classify these strains as *Campylobacter volucris* sp. nov.

Description of *Campylobacter volucris* sp. nov.

Campylobacter volucris (vo.lu'cris. L. gen. n. *volucris* of a bird).

Cells are Gram-negative, non-spore-forming, curved rods. Single polar flagella are observed. All known strains

produce oxidase and catalase and reduce nitrate and selenite. Indoxyl acetate and hippurate are not hydrolysed. Hydrogen sulphide is not produced in triple-sugar iron agar. Alkaline phosphatase-negative. After 3 days of incubation, growth is obtained on blood agar at 37 °C under microaerobic and anaerobic conditions (where growth may be scanty), but not under aerobic conditions. Strains grow at 42 °C but not at 25 °C or at room temperature (18–22 °C). Strains grow on blood agar medium containing 32 mg nalidixic acid, 32 mg cephalothin, 4 mg metronidazole and 32 mg carbenicillin ml^{–1}. Strains grow weakly on MacConkey and potato starch agar media and on media containing 0.1 % sodium fluoride, 0.1 % sodium deoxycholate, 1.0 % ox bile and 0.032 % methyl orange. Strains do not grow on 0.04 % triphenyl-tetrazolium chloride (TTC) medium and, subsequently, reduction of this agent is not observed. No growth on unsupplemented nutrient agar, on a minimal medium or on casein, lecithin or tyrosine media. No growth is observed on media containing 2.0 % NaCl, 1.0 % glycine, 0.02 % safranin, 0.001 % sodium arsenite, 0.1 % potassium permanganate, 0.005 % basic fuchsin, 0.0005 % crystal violet, 0.1 % janus green, 0.02 % pyronin or 64 mg cefoperazone ml^{–1}. Pathogenicity unknown. Strains have been recovered from black-headed gulls (*Larus ridibundus*) in southern Sweden.

The type strain is LMG 24380^T (=CCUG 57498^T), which was isolated from a rectal swab from a black-headed gull in

March 1999. Strains LMG 24379 and LMG 24381, isolated from other individuals of the same species during the same sampling campaign, are reference strains of the species.

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